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A PROCESS FOR THE PREPARATION OF ANTIBODIES BY GENETIC ENGINEERING
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- (57) Claim

1. A gene construct for the preparation of a functional antibody, a functional fragment of an antibody or a fusion protein composed of an antibody domain and other proteins, said gene construct consisting of two or more segments each said segment comprising a gene encoding an individual antibody chain and linked thereto a signal sequence, said two or more segments being coupled to each other so as to form a regulatable operon and so as to enable said genes to undergo simultaneous induction, co-expression and co-secretion in a bacterial host.

2. A process for the in vivo preparation of functional antibodies, functional fragments of the antibodies or fusion proteins composed of antibody domains and other proteins, which comprises coupling genes encoding individual antibody chains each linked to a signal sequence, such that the signal sequence brings about the transport of the antibody chains through the cytoplasmic membrane of a bacterial cell and is then eliminated; bringing about the expression of the genes encoding individual antibody chains to produce functional antibodies, functional fragments of antibodies or fusion proteins composed of antibody domains and other proteins; and isolating the functional antibodies, fragments or fusion proteins from periplasmic space of the bacterium or a medium in which the bacterium is cultured.

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Form 10

COMMONWEALTH OF AUSTRALIA

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Complete Specification for the invention entitled:

A PROCESS FOR THE PREPARATION OF ANTIBODIES BY GENETIC
ENGINEERING

The following statement is a full description of this invention, including the best method of performing it known to : US

Description

**A process for the preparation of antibodies
by genetic engineering**

The expression of antibodies in yeast has been described (C.R. Wood, M.A. Boss, J.H. Kenten, J.E. Calvert, N.A. Roberts and J.S. Emtage, Nature 314, 446 (1985)), but only a very small proportion of the expressed protein proved to be functional. In E. coli, to date it has been possible to obtain antibody proteins only in denatured form (M.A. Boss, J.H. Kenten, C.R. Wood and J.S. Emtage, Nucleic Acids Res. 12, 3791 (1984); S. Cabilly, A.D. Riggs, H. Pande, J.E. Shively, W.E. Holmes, M. Rey, L.J. Perry, R. Wetzel and H.L. Heyneker, Proc. Natl. Acad. Sci. U.S.A. 81, 3273 (1984)). The purification of active antibodies or antibody fragments from yeast or other microorganisms has not been disclosed. Attempts at protein folding have to date resulted in only a very small percentage of correctly folded recombinant antibody proteins. In addition, it is difficult to separate the desired functional proteins from undesired and non-functional proteins, which impedes accurate measurement of binding constants, yields of folding, spectral properties and the like, and use in therapy and industry. Optimization of the folding conditions is thus extremely difficult, especially since there are no proven processes and measurement methods for the refolding.

- 25 The expression of complete functional antibodies or functional binding domains of antibodies in bacterial expression systems has not been disclosed to date, and the prospects of bringing it about have been assessed pessimistically (S.L. Morrison, Science 229, 1202 (1985), M.A. Boss and C.R. Wood, Immunol. Today 6, 12 (1985)).
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Such a system would be very desirable because the genetic

engineering processes have been thoroughly worked out, especially for E. coli, and mass production is facilitated by the rapid growth, which is of considerable economic importance.

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Hence the invention relates to the preparation of functional antibodies, functional fragments thereof, or fusion proteins composed of antibody domains and other proteins in bacteria, preferably Gram-negative bacteria, especially in E. coli. The process according to the invention comprises coupling the genes for the individual chains of the antibody molecule or fragment each to a signal sequence which brings about the transport of the polypeptide chains through the cytoplasmic membrane and which can be split off, bringing about the expression of the gene structures, and isolating the functional protein from the periplasmic space or the medium. Preferred embodiments of this invention are explained in detail hereinafter and defined in the patent claims.

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The coupling of the genes for the individual chains provided with signal sequences preferably takes place in the manner of a regulatable operon system which brings about simultaneous expression by a common control region. In this way, the individual protein chains are expressed together in approximately the stoichiometric ratio, and are transported into the periplasmic space, where joining to form a functional molecule takes place. Proteins are transported out of the cytoplasm by methods known per se, as are described, for example, in European Patent Specification 0,006,694.

Suitable as control region is every suitable regulatable gene regulatory region, for example lac, tac, trp or synthetic sequences. Particularly preferred are regulatory regions which can be reliably turned off.

The proteins are preferably isolated from the periplasmic

space by exerting a mild osmotic shock on the harvested cells, and subjecting the liquid phase obtained by this to concentration by ultrafiltration or precipitation, for example with salts such as ammonium sulfate.

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A "mild" osmotic shock brings about the ejection of the periplasm with, however, the cytoplasmic membrane remaining intact.

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The protein concentrate is, expediently after a dialysis, applied to an adsorbent, advantageously in the form of an affinity column, which is loaded with the appropriate antigen or hapten. The antibody or the functional antibody fragment is then obtained by suitable elution, advantageously with the antigen or hapten.

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In the eukaryotic cell, antibodies are formed in the lumen of the endoplasmic reticulum - probably with the cooperation of disulfide isomerases, proline cis-trans-isomerases and possibly other enzymes or proteins. It was surprising that the bacterial cell is also able to prepare the two chains in approximately the same stoichiometric amount, to transport the two precursor proteins into the periplasmic space or the medium surrounding it, to eliminate the signal sequences correctly, to fold the globular and soluble domains correctly, to form the intramolecular disulfide linkages, and to associate the two chains to give a heterodimer, because it is regarded as improbable that the bacterial cell is equipped with enzymes which are of this nature or have the same effect. Thus, it has surprisingly emerged that, in the case of Gram-negative bacteria, the bacterial periplasm is in this respect functionally equivalent to the lumen of the eukaryotic endoplasmic reticulum.

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The process according to the invention has a number of advantages:
apart from the easy and low-cost large-scale bacterial

fermentation already mentioned, there is direct formation of functional proteins, which thus means that the cleavage of fusion proteins, with subsequent isolation of the desired protein or protein fragment, its oxidation or in vitro refolding, are avoided.

Nor have problems with cellular proteases been found in the process according to the invention. It is, after all, known that, because of these cellular proteases, proteins are normally expressed in bacteria in the form of fusion proteins, especially as insoluble inclusion bodies, which, however, is associated with the stated elaborate further processing steps. In contrast, the separation and purification to homogeneity is rapid and straightforward in the process according to the invention.

Thus the invention permits easy access to antibodies, functional fragments thereof and modified antibodies which differ from the natural antibodies by the insertion, elimination and/or exchange of amino acids. Thus, for example, cysteines can be eliminated or replaced by other amino acids in order to suppress undesired folding. It is possible in just the same way to convert a murine into a human antibody, or to introduce other mutations. Thus, besides the possible pharmacological and industrial applications, access is facilitated for research into antibody structure and function and into the fundamentals of enzymatic catalysis (V. Raso and B.D. Stollar, Biochemistry 14, 584 (1975); V. Raso and B.D. Stollar, Biochemistry 14, 591 (1975); A. Tramontano, K.D. Janda and R.A. Lerner, Science 234, 1566 (1986); S.J. Pollack, J.W. Jacobs and P.G. Schultz, Science 234, 1570 (1986); J. Jacobs, P.G. Schultz, R. Sugawara and M. Powell, J. Am. Chem. Soc. 109, 2174 (1987)).

The invention furthermore permits the application of test systems (assays) directly to the bacterial cell in which the functional antibody is formed, and thus rapid

investigation for possibly mutated antibodies.

Besides the stated variations in the antibody molecule by altering individual, or a few, amino acids, it is also possible to insert into the antibody gene other gene regions, or to exchange parts for non-critical gene regions. It is possible in this way to couple marker enzymes (M.S. Neuberger, G.T. Williams and R.O. Fox, Nature 312, 604 (1984)), toxins (G. Möller (ed.) "Antibody carriers of drugs and toxins in tumor therapy", Immunol. Rev. 62, Munksgaard, Copenhagen (1982)) or immunoglobulin regions of another class (M.S. Neuberger, G.T. Williams, E.B. Mitchell, S.S. Jouhal, J.G. Flanagan, and T.H. Rabbitts, Nature 314, 268 (1985)) or of another species (P.T. Jones, P.H. Dear, J. Foote, M.S. Neuberger, and G. Winter, Nature 321, 522 (1986)) to the antibody molecule.

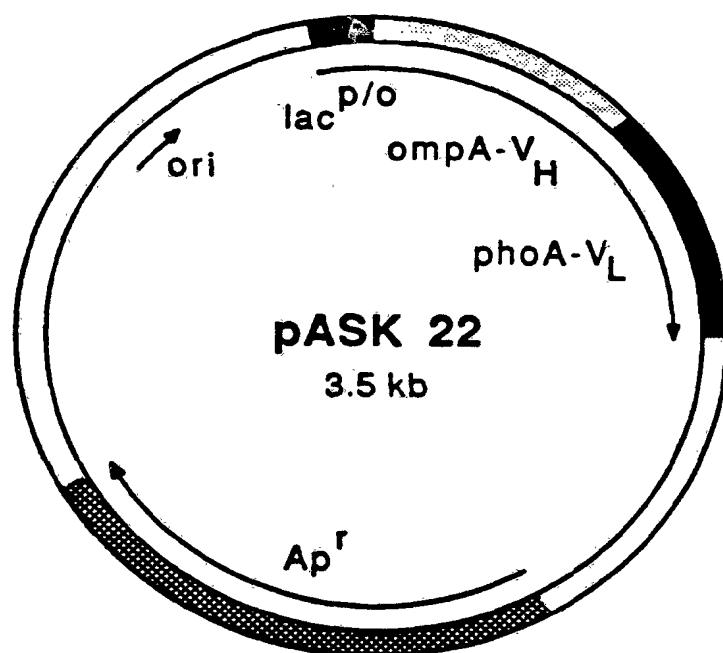
The process according to the invention is explained hereinafter taking the example of the variable domains of the phosphorylcholine-binding antibody myeloma protein McPC603. The three-dimensional structure of this mouse immunoglobulin A is known (D.M. Segal, E.A. Padlan, G.H. Cohen, S. Rudikoff, M. Potter and D.R. Davies, Proc. Natl. Acad. Sci. 71, 4298 (1974)). Synthetic genes for the variable light chain V_L and heavy chain V_H were used. Such synthetic genes are proposed in German Offenlegungsschrift 3,715,033 and published European Patent Application 0,290,005 and published Australian Patent Application 15631/88 (Tables 1 and 2 therein). A particularly expedient embodiment of such synthetic genes is shown in the Table, in which the DNA sequence of the complete expression plasmid is depicted, and the genes for the two chains are emphasized by indicating the amino acids. Codons which are rarely used by *E. coli* were avoided in the construction of these DNA sequences. Furthermore, unique restriction enzyme cleavage sites were incorporated, and account was taken of the secondary structure of the RNA.

In the functional antibody fragment serving as model, each domain has an intramolecular disulfide bridge (from Cys 23 to Cys 94 in V_L and from Cys 22 to Cys 98 in V_H).

- 5 There is no disulfide bridge between the chains, nor is there any free cysteine.

The expression vector used, pASK 22, is depicted diagrammatically in the following formula

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In it the synthetic genes for the V_L and V_H domains are coupled to gene fragments for the bacterial signal sequence of the outer membrane protein A (ompA) on the one hand, and of alkaline phosphatase (phoA) on the other hand. The genes for the two precursor proteins are located in a synthetic operon-like structure downstream of the lac promoter, which ensures that both genes undergo simultaneous induction, coexpression and cosecretion.

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After induction of gene expression, the cells are harvested and exposed to a mild osmotic shock. The liquid

phase obtained by this, which contains the periplasmic proteins, is concentrated by ultrafiltration, dialyzed and applied directly to an affinity column which contains a phosphorylcholine derivative (B. Chesebro and H. Metzger, Biochemistry 11, 766 (1972)) as affinity ligand. Elution with phosphorylcholine results in a homogeneous F_y fragment which is gel electrophoretically homogeneous. It can be deduced from the SDS polyacrylamide gel that the two chains of the purified F_y fragment are present in a molar ratio of 1:1, and the expected molecular weights of the mature proteins (V_H: 13600 D, V_L: 12400 D) are present. To demonstrate correct elimination of the two signal sequences, the six N-terminal amino acids of both chains were sequenced. It emerged that both chains have the correct N-termini for the mature proteins. Thus, both heterologous preproteins have been correctly cleaved by the bacterial signal peptidase, and there is no detectable evidence that imprecise processing or an N-terminal degradation reaction has taken place.

The affinity constant of the recombinant F_y fragment of McPC603 was measured by equilibrium dialysis. The conditions used for this were the same as were applied in the determination of the affinity constant of the natural antibody McPC603 isolated from mouse ascites. The value of $1.21 \pm 0.06 \times 10^5 \text{ M}^{-1}$ found for the F_y fragment is identical, within the accuracy of the experiment, to the value of $1.6 \pm 0.4 \times 10^5 \text{ M}^{-1}$ reported for the natural antibody. The Scatchard binding plot (Ann. N.Y. Acad. Sci. 51, 660 (1949)) is linear, and extrapolation indicates that approximately 1 mol of hapten is bound per mol of F_y fragment. This confirms that there is only one type of binding site per F_y fragment, and that there are no inactive components present in the isolated protein.

Thus, it has emerged, surprisingly, that it is possible to prepare the F_y fragment of the antibody McPC603 as

completely functional and stable protein in E coli. This demonstrates the functional equivalence of the transport into the periplasm of the bacterial cell with the transport into the lumen of the endoplasmic reticulum of the eukaryotic cell. This equivalence has not been disclosed or even suspected, because the bacterial protein which has hitherto been characterized best and which could be defined as soluble heterodimeric protein in the periplasm - E. coli penicillin acylase - is produced by proteolytic processing from a single-chain precursor in the periplasm (G. Schumacher, D. Sizmann, H. Hang, P. Buckel and A. Böck, Nucleic Acids Res. 14, 5713 (1986)). In addition, it has already been pointed out that, according to present knowledge, bacteria have no enzymes or proteins which might in eukaryotes be involved in the folding.

It was also surprising that the F_y fragment of McPC603 has essentially the same affinity constant for phosphorylcholine as has the intact antibody McPC603 itself. This finding is unexpected because the functionality of F_y fragments is controversial in the literature (J. Sen and S. Beychok, Proteins 1, 256 (1986)). It emerges from this that the functionality can be maintained on modification or even complete deletion of the constant domains.

In contrast to the only method used hitherto for the preparation of F_y fragments, namely by proteolysis of an antibody, there are no problems in the process according to the invention with non-functional, incorrectly folded, incorrectly reassociated or chemically modified proteins. Moreover, the preferred isolation process using an antigen- or hapten-loaded adsorbent is also suitable for purifying those F_y fragments obtained by known processes, because such impurities would either not be bound or not be eluted.

Example 1: Preparation of the plasmid pASK22:

The expression plasmid pASK22 is constructed from the large EcoRI-HindIII fragment of pUC12 (C. Yanisch-Perron, J. Vieira, and J. Messing, Gene 33, 103 (1985)), from fragments of the vectors pIN III-OmpA1 (Y. Masui, J. Coleman and M. Inouye in "Experimental manipulation of gene expression", M. Inouye, ed., Academic Press 1983) and pHI61 (H. Inouye, W. Barnes and J. Beckwith, J. Bacteriol. 149, 434 (1982)) as well as various synthetic DNA fragments, in several stages using methods known per se (T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning, Cold Spring Harbor 1982). The complete DNA sequence of the resulting vector pASK22 is depicted in the Table.

The ligation mixture is used to transform competent *E. coli* cells, and the latter are selected for ampicillin resistance. The plasmids with the desired gene structure are characterized by restriction analysis and sequencing of the critical junctions.

Example 2: Preparation of the Fy fragment

A culture of the *E. coli* strain W3110 transformed with pASK22 is cultivated in LB medium containing 100 mg/l ampicillin to an OD₅₅₀ of 0.5. Expression is induced by addition of IPTG to a final concentration of 1mM. After 45 minutes, the cells are harvested by centrifugation at 4000 x g (10 minutes at 4°C). Cell fractionation is carried out by resuspension of the cell pellet in TES buffer (0.2 M tris.HCl, pH 8.0; 0.5 mM EDTA; 0.5 M sucrose) in 10 ml/l of the original culture. The cells are exposed to a mild osmotic shock by addition of 15 ml/l of the original culture of TES buffer which is diluted 1:4 with water and contains 2 mM phosphorylcholine. After the suspension has been incubated on ice for 30 minutes it is centrifuged at 5000 x g for 10 minutes, and the supernatant is subjected to renewed centrifugation at 48000 x g for 15 minutes. The resulting supernatant, which contains all the soluble periplasmic proteins, is

concentrated by ultrafiltration (^(R)AMICON YM5 membrane) to a volume of about 2.5 ml/l of the original culture, and is dialyzed against BBS buffer (0.2 M borate/NaOH, pH 8.0; 0.16 M NaCl). This concentrated solution is
5 applied to an affinity column loaded with a phosphorylcholine derivative (B. Chesebro and H. Metzger, loc.cit.) (1-4 ml of solution per ml of bed volume), which is washed with BBS buffer, and the pure F_y fragment is eluted with a solution of 1mM phosphorylcholine in
10 BBS buffer.

Example 3: Equilibrium dialysis

15 In a dialysis chamber with a volume of 100 µl on each side of the membrane, 50 µl of purified F_y fragment in BBS buffer were placed on one side, and a solution of 50 µl of phosphoryl(methyl-¹⁴C)choline (50 mCi/mmol) in BBS buffer was placed on the other side. The concentration of the F_y fragment determined from the OD₂₀₅ of
20 6.85 was 0.22 mg/ml (R.K. Scopes, Protein Purification - Principles and Practice, Springer-Verlag, New York, 1982, p. 241). After 22 hours at room temperature, equilibrium had been reached, and 20 µl samples of each solution were measured in a scintillation counter (Beckman LS 1801), and
25 the data were subjected to Scatchard plotting (loc. cit.). The affinity constant derived from the gradient of the resulting line is $K_a = 1.21 \pm 0.06 \times 10^5 \text{ M}^{-1}$.

TABLE

DNA sequence of pASK22 with the amino acid sequences of
ompA-V_H and phoA-V_L

1	GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCA	60
	CGCGGGTTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGT	
61	CGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCT	120
	GCTGTCCAAAGGGCTGACCTTTCGCCCGTCACTCGCGTTGCGTTAATTACACTCAATCGA	
121	CACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAAT	180
	GTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACCTTA	
181	TGTGAGCGGATAACAATTTACACAGGAAACAGCTATGACCATGATTACGAATTTCTAGA	240
	ACACTCGCCTATTGTTAAAGTGTGTCCTTGTGCGATACTGGTACTAATGCTTAAAGATCT	
241	TAACGAGGGCAAAAAATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTC	300
	ATTGCTCCCGTTTTTTTACTTTTTCTGTCGATAGCGCTAACGTCACCGTGACCGACCAAAG	
	MetLysLysThrAlaIleAlaIleAlaValAlaLeuAlaGlyPhe -	
301	GCTACCGTAGCGCAGGCCGAAGTTAACTGGTAGAGTCTGGTGGTGGTCTGGTACAGCCG	360
	CGATGGCATCGCGTCCGGCTTCAATTTGACCATCTCAGACCACCACCAGACCATGTCCGC	
	AlaThrValAlaGlnAlaGluValLysLeuValGluSerGlyGlyGlyLeuValGlnPro -	
361	GGTGGATCCCTGCGTCTGTCTTGCGCTACCTCAGGTTTCACCTTCTCTGACTTCTACATG	420
	CCACCTAGGGACGCAGACAGAACGCGATGGAGTCCAAAGTGAAGAGACTGAAGATGTAC	
	GlyGlySerLeuArgLeuSerCysAlaThrSerGlyPheThrPheSerAspPheTyrMet -	
421	GAGTGGGTACGTCAGCCCCCGGGTAAACGTCTCGAGTGGATCGCAGCTAGCCGTAACAAA	480
	CTCACCCATGCAGTCGGGGGCCCATTTGCAGAGCTCACCTAGCGTCGATCGGCATTGTTT	
	GluTrpValArgGlnProProGlyLysArgLeuGluTrpIleAlaAlaSerArgAsnLys -	
481	GGTAACAAGTATACCACCGAATACAGCGCTTCTGTTAAAGGTCGTTTCATCGTTTCTCGT	540
	CCATTGTTTCATATGGTGGCTTATGTGCGGAAGACAATTTCCAGCAAAGTAGCAAAGAGCA	
	GlyAsnLysTyrThrThrGluTyrSerAlaSerValLysGlyArgPheIleValSerArg -	
541	GACACTAGTCAATCGATCCTGTACCTGCAGATGAATGCATTGCGTGCTGAAGACACCGCT	600
	CTGTGATCAGTTAGCTAGGACATGGACGCTACTTACGTAACGCACGACTTCTGTGGCGA	
	AspThrSerGlnSerIleLeuTyrLeuGlnMetAsnAlaLeuArgAlaGluAspThrAla -	

501 ATCTACTACTGCGCGCGTAACACTACTATGGCAGCACTTGGTACTTCGACGTTTGGGGTGCA 660
 -----+-----+-----+-----+-----+-----+
 TAGATGATGACGCGCGCATTGATGATACCGTCGTGAACCATGAAGCTGCAAACCCACGT
 IleTyrTyrCysAlaArgAsnTyrTyrGlySerThrTrpTyrPheAspValTrpGlyAla -
 661 GGTACCACCGTTACCGTTTCTTCTTGATAACATGGAGAAAATAAAGTGAACAAAGCACT 720
 -----+-----+-----+-----+-----+-----+
 CCATGGTGGCAATGGCAAAGAAGAACTATTGTACCTCTTTTATTTCACTTTGTTTCGTGA
 GlyThrThrValThrValSerSerEnd MetLysGlnSerThr -
 721 ATTGCACTGGCACTCTTACCGTTACTGTTTACCCCTGTGACAAAAGCCGATATCGTTATG 780
 -----+-----+-----+-----+-----+-----+
 TAACGTGACCGTGAGAATGGCAATGACAAATGGGGACACTGTTTTCGGCTATAGCAATAC
 IleAlaLeuAlaLeuLeuProLeuLeuPheThrProValThrLysAlaAspIleValMet -
 781 ACCCAGTCTCCGAGCTCTCTGTCTGTATCTGCAGGTGAACGTGTTACCATGTCTTGCAA 840
 -----+-----+-----+-----+-----+-----+
 TGGGTCAGAGGCTCGAGAGACAGACATAGACGTCCACTTGCACAATGGTACAGAACGTTT
 ThrGlnSerProSerSerLeuSerValSerAlaGlyGluArgValThrMetSerCysLys -
 841 TCTTCTCAGTCTCTGCTGAACCTCTGGTAACCAGAAAACTTCTTGGCGTGGTATCAGCAA 900
 -----+-----+-----+-----+-----+-----+
 AGAAGAGTCAGAGACGACTTGAGACCATTGGTCTTTTGAAGGACCGCACCATAGTCGTT
 SerSerGlnSerLeuLeuAsnSerGlyAsnGlnLysAsnPheLeuAlaTrpTyrGlnGln -
 901 AAGCCTGGCCAAACCGCCGAAACTGCTGATCTACGGTGGCTCGACCCGTGAATCTGGTGT 960
 -----+-----+-----+-----+-----+-----+
 TTCGGACCGGTTGGCGGCTTTGACGACTAGATGCCACGCAGCTGGGCACTTAGACCACAA
 LysProGlyGlnProProLysLeuLeuIleTyrGlyAlaSerThrArgGluSerGlyVal -
 961 CCGGACCGTTTTACCGGTAGCGGTAGCGGTACCGACTTCACTCTGACCATCTCTTCTGTA 1020
 -----+-----+-----+-----+-----+-----+
 GGCCTGGCAAAATGGCCATCGCCATCGCCATGGCTGAAGTGAGACTGGTAGAGAAGACAT
 ProAspArgPheThrGlySerGlySerGlyThrAspPheThrLeuThrIleSerSerVal -
 1021 CAGGCTGAAGATCTGGCTGTTTACTACTGTCAAACGACCACTCTTACCCGCTGACCTTT 1080
 -----+-----+-----+-----+-----+-----+
 GTCCGACTTCTAGACCGACAAATGATGACAGTTTTGCTGGTGAGAATGGGCGACTGGAAA
 GlnAlaGluAspLeuAlaValTyrTyrCysGlnAsnAspHisSerTyrProLeuThrPhe -
 1081 GCGCGCGGCACCAAACCTGGAACCTGAAGCGCGCTTGATAAGCTTGGCACTGGCCGTCGTT 1140
 -----+-----+-----+-----+-----+-----+
 CCGCGCGCGTGGTTTGACCTTGACTTCGCGCGAACTATTGGAACCGTGACCGGCAGCAAA
 GlyAlaGlyThrLysLeuGluLeuLysArgAlaEnd
 1141 TACAACGTCGTGACTGGGAAAACCTGGCGTTACCCAACTTAATCGCCTTGACGACATC 1200
 -----+-----+-----+-----+-----+-----+
 ATGTTGCAGCACTGACCCTTTTGGGACCGCAATGGGTTGAATTAGCGGAACGTCGTGTAG
 1201 CCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGT 1260
 -----+-----+-----+-----+-----+-----+
 GGGGAAAGCGGTCGACCGCATTATCGCTTCTCCGGGCGTGGCTAGCGGGAAGGGTTGTCA

1261 TCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTCTCCTTACGCATCTGTGCG
-----+-----+-----+-----+-----+-----+ 1320
ACGCGTCGGACTTACCGCTTACCGCGGACTACGCCATAAAAGAGGAATGCGTAGACACGC

1321 GTATTTACACCGCATATGGTGCACCTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAA
-----+-----+-----+-----+-----+-----+ 1380
CATAAAGTGTGGCGTATACCACGTGAGAGTCATGTTAGACGAGACTACGGCGTATCAATT

1381 GCCAGCCCGACACCCGCCAACCCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGG
-----+-----+-----+-----+-----+-----+ 1440
CGGTCGGGGCTGTGGGCGGTGTGGGCGACTGCGCGGJACTGCCCGAACAGACGAGGGCC

1441 CATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCAC
-----+-----+-----+-----+-----+-----+ 1500
GTAGGCGAATGTCTGTTTCGACACTGGCAGAGGCCCTCGACGTACACAGTCTCCAAAAGTG

1501 CGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTA
-----+-----+-----+-----+-----+-----+ 1560
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1561 ATGTCATGATAATAATGGTTTCTTAGACGTGAGTGGCACTTTTTCGGGGAAATGTGCGCG
-----+-----+-----+-----+-----+-----+ 1620
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1621 GAACCCCTATTTGTTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAAT
-----+-----+-----+-----+-----+-----+ 1680
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1681 AACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCC
-----+-----+-----+-----+-----+-----+ 1740
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1741 GTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCCTTCTGTTTTTGCTCACCCAGAAA
-----+-----+-----+-----+-----+-----+ 1800
CACAGCGGGAATAAGGGAAAAAACGCCGTAAACCGGAAGGACAAAAACGAGTGGGTCTTT

1801 CGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATCGAAC
-----+-----+-----+-----+-----+-----+ 1860
GCGACCACTTTTCATTTTCTACGACTTCTAGTCAACCCACGTGCTCACCCAATGTAGCTTG

1861 TGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAAGCTTTTCCAATGA
-----+-----+-----+-----+-----+-----+ 1920
ACCTAGAGTTGTGCGCCATTCTAGGAACCTCTCAAAGCGGGGCTTCTTGCAAAGGTTACT

1921 TGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAG
-----+-----+-----+-----+-----+-----+ 1980
ACTCGTGAAAATTTCAAGACGATACACCGCGCCATAATAGGGCATAACTGCGGCCCGTTC

1981 AGCAACTCGGTGCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCACTCA
-----+-----+-----+-----+-----+-----+ 2040
TCGTTGAGCCAGCGGCGTATGTGATAAGAGTCTTACTGAACCACTCATGAGTGGTCACT

2041 CAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCA 2100
 -----+-----+-----+-----+-----+-----+-----+
 GTCTTTTCGTAGAAATGCCTACCGTACTGTCATTCTCTTAATACGTCACGACGGTATTGGT

2101 TGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAA 2160
 -----+-----+-----+-----+-----+-----+-----+
 ACTCACTATTGTGACGCCGGTTGAATGAAGACTGTTGCTAGCCTCCTGGCTTCCTCGATT

2161 CCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGC 2220
 -----+-----+-----+-----+-----+-----+-----+
 GGCGAAAAAACGTGTTGTACCCCTAGTACATTGAGCGGAAGTAGCAACCCTTGGCCTCG

2221 TGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAA 2280
 -----+-----+-----+-----+-----+-----+-----+
 ACTTACTTCGGTATGGTTTGCTGCTCGCACTGTGGTGCTACGGACATCGTTACCGTTGTT

2281 CGTTGCGCAAACCTATTAACCTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAG 2340
 -----+-----+-----+-----+-----+-----+-----+
 GCAACGCGTTTGATAATTGACCGCTTGATGAATGAGATCGAAGGGCCGTTGTTAATTATC

2341 ACTGGATGGAGGCGGATAAAGTTGCAGGACCACCTTCTGCGCTCGGCCCTTCCGGCTGGCT 2400
 -----+-----+-----+-----+-----+-----+-----+
 TGACCTACCTCCGCCTATTTCAACGTCTTGGTGAAGACGCGAGCCGGAAGGCCGACCGA

2401 GGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCAC 2460
 -----+-----+-----+-----+-----+-----+-----+
 CCAAATAACGACTATTTAGACCTCGGCCACTCGCACCCAGAGCGCCATAGTAACGTCGTG

2461 TGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAA 2520
 -----+-----+-----+-----+-----+-----+-----+
 ACCCCGGTCTACCATTCTGGGAGGGCATAGCATCAATAGATGTGCTGCCCTCAGTCCGTT

2521 CTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGT 2580
 -----+-----+-----+-----+-----+-----+-----+
 GATACCTACTTGCTTTATCTGTCTAGCGACTCTATCCACGGAGTGACTAATTCGTAACCA

2581 AACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTAAAACTTCATTTTTAAT 2640
 -----+-----+-----+-----+-----+-----+-----+
 TTGACAGTCTGGTTCAAATGAGTATATATGAAATCTAACTAAATTTGAAGTAAAAATTA

2641 TTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTG 2700
 -----+-----+-----+-----+-----+-----+-----+
 AATTTTCCTAGATCCACTTCTAGGAAAACTATTAGAGTACTGGTTTTAGGGAATTGCAC

2701 AGTTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATC 2760
 -----+-----+-----+-----+-----+-----+-----+
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2761 CTTTTTTTCTGCGGTAATCTGCTGCTTGCAAACAAAAAACCCACCGCTACCAGCGGTGG 2820
 -----+-----+-----+-----+-----+-----+-----+
 GAAAAAAGACGCGCATTAGACGACGAACGTTGTTTTTTTTGGTGGCGATGGTCGCCACC

2821 TTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAAGTGGCTTCAGCAGAG 2880
 -----+-----+-----+-----+-----+-----+-----+
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2881 CGCAGATACCAAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT 2940
 -----+-----+-----+-----+-----+-----+-----+
 GCGTCTATGGTTTATGACAGGAAGATCACATCGGCATCAATCCGGTGGTGAAGTTCCTGA

2941 CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTG 3000
 -----+-----+-----+-----+-----+-----+-----+
 GACATCGTGGCGGATGTATGGAGCGAGACGATTAGGACAATGGTCACCGACGACGGTCAC

3001 GCGATAAGTCGTGCTTACCGGGTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGC 3060
 -----+-----+-----+-----+-----+-----+-----+
 CGCTATTCAGCACAGAATGGCCCAACCTGAGTTCGTCTATCAATGGCCTATTCCGCGTCG

3061 GGTCCGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCG 3120
 -----+-----+-----+-----+-----+-----+-----+
 CCAGCCCGACTTGCCCCCAAGCACGTGTGTGCGGTGGAACCTCGCTTGCTGGATGTGGC

3121 AACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGG 3180
 -----+-----+-----+-----+-----+-----+-----+
 TTGACTCTATGGATGTGCGACTCGATACTCTTTCGCGGTGCGAAGGGCTTCCCTCTTTCC

3181 CGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAG 3240
 -----+-----+-----+-----+-----+-----+-----+
 GCCTGTCCATAGGCCATTGCGCGTCCAGCCTTGCTCTCTCGCGTGCTCCCTCGAAGGTC

3241 GGGGAAACGCCTGGTATCTTTATAGTCTGTGCGGGTTTCGCCACCTCTGACTTGAGCGTC 3300
 -----+-----+-----+-----+-----+-----+-----+
 CCCCTTTGCGGACCATAGAAATATCAGGACAGCCCAAAGCGGTGGAGACTGAACTCGCAG

3301 GATTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCT 3360
 -----+-----+-----+-----+-----+-----+-----+
 CTAAAAACACTACGAGCAGTCCCCCGCTCGGATACCTTTTTCGGGTGCTTGCGCCGGA

3361 TTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCC 3420
 -----+-----+-----+-----+-----+-----+-----+
 AAAATGCCAAGGACCGGAAAACGACCGGAAAACGAGTGTACAAGAAAGGACGCAATAGGG

3421 CTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCC 3480
 -----+-----+-----+-----+-----+-----+-----+
 GACTAAGACACCTATTGGCATAATGGCGGAAACTCACTCGACTATGGCGAGCGGCGTCCG

3481 GAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGA 3523
 -----+-----+-----+-----+-----+-----+-----+
 CTTGCTGGCTCGCGTCGCTCAGTCACTCGCTCCTTCGCCTTCT

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A gene construct for the preparation of a functional antibody, a functional fragment of an antibody or a fusion protein composed of an antibody domain and other proteins, said gene construct consisting of two or more segments each said segment comprising a gene encoding an individual antibody chain and linked thereto a signal sequence, said two or more segments being coupled to each other so as to form a regulatable operon and so as to enable said genes to undergo simultaneous induction, co-expression and co-secretion in a bacterial host.

2. A process for the in vivo preparation of functional antibodies, functional fragments of the antibodies or fusion proteins composed of antibody domains and other proteins, which comprises coupling genes encoding individual antibody chains each linked to a signal sequence, such that the signal sequence brings about the transport of the antibody chains through the cytoplasmic membrane of a bacterial cell and is then eliminated; bringing about the expression of the genes encoding individual antibody chains to produce functional antibodies, functional fragments of antibodies or fusion proteins composed of antibody domains and other proteins; and isolating the functional antibodies, fragments or fusion proteins from periplasmic space of the bacterium or a medium in which the bacterium is cultured.

3. A process for the in vivo preparation of a functional antibody, a functional fragment of the antibody or a fusion protein comprising an antibody domain, comprising placing on a single plasmid a first DNA sequence consisting of:

- (a) a first nucleotide sequence that encodes an antibody heavy chain or a functional fragment thereof, and
- (b) a first signal sequence derived from a prokaryote, and

a second DNA sequence consisting of:

- (a) a second nucleotide sequence that encodes an antibody light chain or a functional fragment thereof, and
- (b) a second signal sequence derived from the prokaryote;



bringing about expression of the first and second nucleotide sequences in a bacterium to produce a functional antibody, a functional fragment of the antibody or a fusion protein comprising an antibody domain, and isolating the functional antibody, functional fragment or the fusion protein from periplasmic space of the bacterium or from a medium in which the bacterium is cultured.

4. The process as claimed in claims 2 or 3, wherein the bacterium is a Gram-negative bacterium.

5. The process as claimed in claim 4, wherein the bacterium is *E. coli*.

6. The process as claimed in claims 2 - 5, wherein the genes are coupled in the form of a regulatable operon system.

7. The process as claimed in one or more of the preceding claims, wherein, in order to isolate the functional protein from the periplasmic space, the bacteria which have been separated off are exposed to an osmotic shock such that the periplasm is ejected but the cytoplasm remains in tact, the liquid phase obtained by this is enriched by centrifugation, and the desired proteins are obtained from this concentrate.

8. The process as claimed in one or more of the preceding claims, wherein the solution containing the functional protein is applied to an absorbent loaded with the appropriate antigen or hapten, and the desired proteins are isolated by elution with a solution containing this antigen or hapten.

9. A functional antibody, a functional fragment of an antibody or a fusion protein composed of an antibody domain and other proteins prepared from a gene construct as claimed in claim 1.



10. A functional antibody, a functional fragment of an antibody or a fusion protein composed of an antibody domain and other proteins prepared according to the process of any one of claims 2 - 8.

DATED this 11th day of March 1992.

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